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Comparison and evaluation of two rapid diagnostic assays for the identification of Mycoplasma species

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Comparison and evaluation of two rapid diagnostic assays for the identification of *Mycoplasma* species

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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NOMENCLATURE

DNA	Deoxyribonucleic acid
GC	Guanine/cytosine
HRM	High-resolution melting
HRM-PCR	High-resolution melting polymerase chain reaction
IGSR	Intergenic spacer region
IHC	Immunohistochemistry
MALDI-TOF	Matrix-assisted laser desorption/ionization time-of-flight
MLST	Multilocus sequence typing
MLVA	Multiple-locus variable number tandem repeat analysis
NAD	Nicotinamide adenine dinucleotide
PCR	Polymerase chain reaction
PPLO	Pleuropneumoniae-like organism
qPCR	Real-time polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
SNP	Single nucleotide polymorphism
TCA	Tricarboxylic acid
T_M	Melting temperature

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ABSTRACT

Members of the class Mollicutes are unique among bacteria in that they are unable to synthesize a cell wall. These organisms are also difficult to culture and very slow-growing with some species requiring up to 40 days to culture. Many of these bacteria are also significant pathogens in humans and animals, so rapid identification of isolates is a necessary diagnostic step. Many different tests are used for identification of pathogenic Mollicutes (primarily *Mycoplasma* and *Ureaplasma* species), however most tests are not cost or time efficient. Two relatively recent diagnostic tools are matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and high-resolution melting polymerase chain reaction (HRM-PCR). Nine isolates of *Mycoplasma bovis*, a pathogen in cattle, were grown and used first in a MALDI-TOF assay and then in an HRM-PCR assay utilizing universal primers for the 16S-23S rRNA intergenic spacer region (IGSR) of *Mycoplasma* species. The HRM-PCR assay compared the melt curve profile of the control isolate to the other eight for species determination. Both assays were able to successfully identify all nine isolates. Next, a new HRM-PCR assay also targeting the 16S-23S rRNA IGSR was developed and compared to the original assay. Reference and field strains of six *Mycoplasma* species were cultured along with reference strains for two additional *Mycoplasma* species and one *Ureaplasma* species. All of these isolates were tested in both the first (HRM-PCR-1) and second (HRM-PCR-2) HRM assay, and melt curve profiles of the field isolates were compared to the controls for identification. HRM-PCR-1 failed to amplify *Mycoplasma gallisepticum* and *Ureaplasma diversum* and was unable to identify the majority of *Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae* isolates. HRM-PCR-2 identified the majority of *M. hyorhinis* and all *Mycoplasma canis* isolates, but failed to identify *M. gallisepticum*. All isolates of *Mycoplasma bovis* and *Mycoplasma hyopneumoniae* were

successfully identified in both methods. HRM-PCR is a promising tool for identification, but more validation is necessary to provide consistent and accurate results. Future work should focus on expanding the MALDI-TOF database and testing different species in the HRM-PCR assays. Additionally, the use of HRM-PCR directly on tissue samples should be explored.

CHAPTER 1: THESIS ORGANIZATION

This thesis is organized into five chapters. Chapter 1 contains the outline of the thesis. Chapter 2 reviews general information about the class Mollicutes, its significance, and diagnostic methods that are used with these organisms. Chapter 3 contains research comparing a MALDI-TOF assay to a high-resolution melting PCR assay for *Mycoplasma* species identification. Chapter 4 also contains research, comparing two high-resolution melting PCR assays for the identification of *Mycoplasma* species. Chapter 5 serves as a general conclusion.

CHAPTER 2: REVIEW OF THE MOLLICUTES AND DIAGNOSTIC METHODS

Introduction

Members of the class Mollicutes are among the smallest free-living organisms, with diameters as small as 0.3 μm. Mollicutes have a variety of distinctive features such as their small genomes, the low guanine (G) and cytosine (C) content of their genomes and their fastidious nature (Razin, 1983). These organisms are thought to be derived from Gram-positive bacteria, and through extensive genomic reduction have developed their most significant feature: the complete lack of a cell wall (Razin et al., 1998).

Members of the Mollicutes are known for not only being some of the smallest free-living organisms in diameter, but also in genome size. For example, *Mycoplasma genitalium* has a genome of only 580,070 bp (Fraser et al., 1995). *Ureaplasma parvum* has a genome size of only 727,289 bp (Wu et al., 2014). In contrast, *E. coli* K-12 has a genome size of 4,639,221 bp, approximately 8 times larger than that of *M. genitalium* (Blattner et al., 1997). The genomes of Mollicutes differ from conventional bacteria not only in size but also in composition. The GC-content of *E. coli* K-12 is 50.8% (Blattner et al., 1997), while that of *M. genitalium* is 32% (Fraser et al., 1995) and that of U. parvum is 25.6% (Wu et al., 2014). This lower G+C-content is characteristic of the Mollicutes. The G+C-content of a bacterium is reflective of its capabilities as an organism. In general, G+C-content is directly correlated with genome size and the organism's adaptability, with a higher genome size and G+C-content typically exhibiting a greater ability to adapt to different environments (Mann and Chen, 2010). As these organisms are generally host-specific and fastidious, their small genomes and low GC-content are consistent with this observation.

Mollicutes lack a large portion of biochemical processes that other bacteria possess. For example, *Mycoplasma hyopneumoniae, Mycoplasma synoviae,* and *Mycoplasma mycoides* lack the capabilities for *de novo* nucleotide synthesis (Mitchell and Finch, 1977; Arraes et al., 2007). Additionally, *M. synoviae* is one of the few Mollicutes capable of converting citrate to acetate and oxaloacetate, a step in the reductive TCA cycle, and not a single amino acid is synthesized by *M. hyopneumoniae* (Arraes et al., 2007). Because of these and additional biochemical deficiencies, Mollicutes are generally quite fastidious. Culturing *Mycoplasma* species and other members of the Mollicutes is a tedious and difficult process. Sterol is required for the growth of most Mollicutes, and so complex media containing animal serum is utilized (Rodwell and Abbot, 1961). Culturing times vary, but can take anywhere from 3 to 40 days depending on the species (Lauerman, 1994; Jensen et al., 1996).

Another common feature of the Mollicutes is the fried-egg morphology of colonies when grown on solid media. The colonies have a central area in which the cells grow into the agar with a peripheral zone that spreads out along the surface of the agar, resulting in a colony that resembles a fried egg (Razin and Oliver, 1961). This is not unique to the Mollicutes, however. L-forms of bacteria are phases in which the cells produce either a partial or no cell wall, and many bacteria are capable of exhibiting an L-form. (Glover et al., 2009). When these L-forms are grown on agar plates, they can also exhibit a fried-egg morphology, and the L-form of a bacterium can be induced by the introduction of antibiotics such as penicillin and other β-lactams (Razin and Oliver, 1961). These antimicrobials are frequently used in the culturing of Mollicutes because they will prevent the growth of conventional bacteria while allowing Mollicutes to grow as they lack the cell wall targets of these antibiotics. This creates a challenge when attempting to

grow and identify species within the Mollicutes, as an L-form can be mistaken as a *Mycoplasma* species.

Diagnostic Needs

Veterinary Pathogens

Identifying species of Mollicutes is necessary for diagnostics, as many are human, animal, and plant pathogens. In human and animal hosts, Mollicutes tend to colonize the mucosal areas of the body including the respiratory tract and the urogenital tract (Trachtenberg, 2005). These bacteria can exist as commensals, but more frequently they are pathogens in their hosts (Razin, 1983). As animal pathogens, *Mycoplasma* species collectively cause a variety of diseases such as pneumonia, arthritis, conjunctivitis, mastitis, otitis media, and hemolytic anemia (Lauerman, 1994; Wang et al., 2017). Usually these infections are not severe, but they increase the risk of additional infections or severity of disease in co-infected animals (Staugas and Martin, 1985; Thacker et al., 1999). *Ureaplasma* species are also known to cause disease in animals. These infections occur in the urogenital tract and can cause abortions, infertility, and endometritis (Ruhnke, 1994; Waites et al., 2005).

As previously stated, *Mycoplasma* and *Ureaplasma* species are veterinary pathogens, and can cause various diseases. In swine, for example, *M. hyopneumoniae* is known to cause porcine enzootic pneumonia which has low mortality but high morbidity in infected animals. The disease causes depletion of the ciliated epithelial cells of the lower respiratory tract and is immunomodulatory, increasing the susceptibility of the pigs to secondary infections (DeBey and Ross, 1994). Secondary infections are also more common in infections with hemotropic *Mycoplasma* species. While originally thought to be unrelated, these blood parasites were discovered to be members of the Mollicutes after the emergence of better molecular tools for

phylogenetic analyses (Messick, 2004). One such hemotropic organism is *Mycoplasma ovis*. This bacterium infects small ruminants such as sheep and goats and usually causes a latent infection. Infected animals will become more prone to secondary infections with other bacterial pathogens, however, increasing the risk of severe disease (Rjeibi et al., 2015). *Mycoplasma canis* is a common commensal in dogs, but it is also an opportunistic pathogen of the urogenital tract, causing urinary tract infections, prostatitis, and other infections (L'Abee-Lund et al., 2003). An association has also been shown between the presence of *M. canis* in canine brain tissues and granulomatous meningoencephalitis or necrotizing meningoencephalitis, conditions that cause seizures, blindness, and often death (Michaels et al., 2016). In cattle, *Ureaplasma diversum* can complicate breeding practices. When infected, cows may become infertile or undergo spontaneous abortions. Calves that are born from infected cows may develop weak-calf syndrome, often resulting in a weakened immune system and low birth weight (Takasu et al., 2008).

Human Pathogens

There are also significant human pathogens among the Mollicutes. *Mycoplasma pneumoniae* is the etiological agent of primary atypical pneumonia in humans, and can cause otitis media, pharyngitis and the formation of abscesses as well (Atkinson et al., 2008). In addition to pneumonia, *M. pneumoniae* has also been identified as a risk factor for asthma in patients with a chronic infection (Sutherland et al., 2004). Also pathogenic in humans is *M. genitalium*. This organism can be sexually-transmitted and causes urethritis in men and cervicitis, infertility, and pelvic inflammatory disease in women (Manhart, 2017).

Cell Culture Contaminants

In cell culture, Mollicutes such as *Mycoplasma* and *Acholeplasma* species are among the most frequent contaminants. Based on routine testing from the 1970s to 1994, up to 15% of all cell cultures in the United States may be contaminated with Mollicutes at any given time (Ryan, 2008). There are multiple sources from which the contaminant may be introduced; cells from the persons handling the cultures, the serum used in the cell culture media, and use of improperly sterilized tools are all suggested origins (Drexler and Uphoff, 2002). While antibiotics are frequently used in cell culture, they may not always help as any β-lactam antibiotics will have no effect on the Mollicutes.

The effects that Mollicutes have on cell cultures depend heavily on the metabolic capabilities of the contaminant, and so can vary greatly. *Mycoplasma gallisepticum* can induce plaque formation in cell monolayers similar to those of viral agents, making it difficult to determine if a there truly is a contaminant when growing viruses. Additionally, the cytopathic effects of the contaminant may be severe or unapparent, or may decrease or increase viral yields (Barile and Grabowski, 1978). Because of the unpredictable effects of *Mycoplasma* and *Acholeplasma* contaminants, rigorous testing is necessary to ensure the purity of cell cultures.

Economic and Ecological Impact

Mycoplasma and *Ureaplasma* species cause many different kinds of infections in a variety of hosts and can be extremely costly to the animal industry. In 2014 it was estimated that infections of *M. hyopneumoniae* cost the United States' swine industry up to $400 million each year (Holtkamp, 2014). Two other species*, Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae,* are also known to cause disease in swine and contribute to up to 43% of all losses due to lameness in pigs (Wilberts et al., 2015). Cattle can also experience mycoplasma

infections. *Mycoplasma bovis* causes respiratory disease, mastitis, and arthritis in cattle and causes annual losses of up to $32 million to the beef industry and $108 million to the dairy industry (Maunsell et al., 2011). In the poultry industry, *M. gallisepticum* causes chronic respiratory disease and has been shown to decrease weight gain and production in egg layers. A 1987 study showed a total loss of approximately $7.3 million to egg producers in southern California (Mohammed et al., 1987). *Mycoplasma gallisepticum* infections are still a problem today, though to the authors' knowledge no recent studies on its economic impact have been conducted.

Mycoplasma infections are not just a problem in agriculture but are also a problem for wildlife. *Mycoplasma gallisepticum,* long-considered a pathogen in chickens and turkeys, also causes disease in songbirds. It emerged as a significant pathogen of the house finch in 1994 and is now known to cause severe conjunctivitis in these birds (Luttrell et al., 1998). In fact, within three years after it was first identified, *M. gallisepticum* devastated house finch populations, causing a decline of up to 60% in the eastern United States (Badyaev, 2012).

Identification and Characterization Methods

Because *Mycoplasma* and *Ureaplasma* species are significant pathogens and cell culture contaminants, methods of identification are vital for diagnostic purposes. There are many different identification methods used for these organisms, from traditional biochemical or serological tests to more recent molecular methods such as polymerase chain reaction (PCR).

Biochemical and Serological Tests

As previously stated, when grown on agar, most members of the Mollicutes form a distinct fried-egg colony morphology (Razin and Oliver, 1961). Monitoring the growth of a suspect *Mycoplasma* or *Ureaplasma* species and looking for the correct colony morphology may

be sufficient to identify the organism as a member of the Mollicutes. However since L-forms of bacteria can also form this same morphology, confirmation is essential to ensure that a Mollicutes suspect is not an L-form. L-forms will revert back to their cell walled form eventually, so a series of five passages in media without an antibiotic is considered sufficient to rule them out (Goll, 1994).

Once a culture is obtained and confirmed as a member of the Mollicutes, there are numerous biochemical tests that can be performed to identify the species isolated. Glucose fermentation, a vital first test for ruling out a large percentage of *Mycoplasma* suspects, can be determined through the use of a pH indicator in the media. When the bacterium is able to ferment glucose, the media will become more acidic, resulting in a lower pH and subsequent change in the media color (Goll, 1994). An arginine test is another important tool for identifying Mollicutes biochemically, as most of the *Mycoplasma* species that are not able to ferment glucose are able to hydrolyze arginine (Goll, 1994). To differentiate a *Mycoplasma* species from a *Ureaplasma* species, two different media can be used when culturing: one with added urea and one without, as *Ureaplasma* species require urea to grow (Shepard, 1983). After these tests are complete, further biochemical media can be used to determine the species.

Multiple kinds of serological tests can also be used to identify Mollicutes. If a certain species is suspected of causing disease in an animal, culture can be done in duplicate with one set of media containing antisera to the suspect organism. Growth in the absence of antisera but not in the presence in theory confirms the identity of the organism. This is referred to as a growth inhibition test. In practice, however, inaccurate identification can occur due to cross-reactivity of the antibodies (Clyde, 1964). A second type of serological test is one utilizing immunofluorescence. For this assay, fluorescently labeled antibodies to a certain organism are

used. A fluorescent signal, when viewed with a fluorescent microscope, is considered positive for the organism of interest (Del Giudice et al., 1967). A third serological test is immunohistochemistry (IHC) in which a culture of the suspect organism is not needed. Instead, the antibodies are applied directly to the tissue and visualized under a microscope through the use of a tag, often streptavidin-biotin, and a fluorescent probe (Opriessnig et al., 2004).

Molecular Diagnostic Tests

While biochemical tests are considered the gold standard, they require multiple cultures to assess, and can take weeks to complete. Serological tests can be very sensitive, but maintaining antibodies specific to every *Mycoplasma* species that might be grown in a diagnostic laboratory is very expensive. To overcome some of these barriers, PCR assays have been developed for Mollicutes, and these are rapid and relatively inexpensive. These tests target specific regions of DNA and do not need to be used on a bacterial culture (Yang and Rothman, 2004). Conventional PCR targets a specific region of DNA and, after amplification, the product is detected through agarose gel electrophoresis. If an appropriately-sized band is present, then the tissue sample or culture is considered positive (Yang and Rothman, 2004). For Mollicutes, PCR assays frequently target the 16S rRNA genes of the organisms but may target other regions of the chromosome as well (Robertson et al., 1993; Chavez Gonzalez et al., 1995; Jensen et al., 2003).

Real-time PCR (qPCR) targets a much smaller region of DNA than conventional PCR and utilizes a fluorophore in the PCR reaction which eliminates a subsequent detection step. If the amount of fluorescence reaches a certain level, the sample is considered positive for the organism of interest. If quantitation of the organism is desired, serial dilutions of a standard with a known quantity of DNA can be used to generate a standard curve from which quantities in an unknown sample can be calculated. Two main types of qPCR exist: those using hybridization

probes and those using DNA intercalating dyes. The former assays use a short oligonucleotide probe containing a fluorophore in addition to forward and reverse primers. When the probe is degraded during the assay as a function of the DNA polymerase, the fluorophore is released and emits light (Holland et al., 1991). Fluorescent dyes that intercalate into DNA are also used for qPCR. These are more prone to false positives than hybridization probe assays, but are also less expensive because specific probes are not needed (Ponchel et al., 2003). If a melt step follows the qPCR, specificity can be improved by comparing the melting temperature (T_M) of the positive control to the sample (Downey, 2015). Both types of real-time PCR assays have been used for detection of *Mycoplasma* and *Ureaplasma* species (Dubosson et al., 2004; Xiao et al., 2010; Gomes Neto et al., 2015).

Molecular Characterization

In addition to identification, characterization methods are often used with *Mycoplasma* species during the diagnostic process. Two common tools used today for molecular characterization of *Mycoplasma* species are multi-locus sequence typing (MLST) and multiple locus variable-number tandem repeat analysis (MLVA). One major use of these typing tools is for molecular epidemiology. By following the movement of similar strains, producers can locate the source of an outbreak and implement measures for minimizing or preventing outbreaks in the future.

In MLST, between three and seven genes are characterized. Typically housekeeping genes are used, as these mutate very slowly and so are better candidates for strain typing (Maiden, 2006). After performing PCR on the selected genes, the products are sequenced, and the different isolates are classified based on the various combinations of the different sequences. One major downfall of this type of assay is that the sequence types are completely dependent on

the targets used, so to compare isolates, the exact same typing scheme must be used. To improve the universal utilization of these assays, the database PubMLST is available to share typing schemes globally, and as of 2018, schemes for seven different *Mycoplasma* species have been uploaded (Oxford). Additionally, MLST relies on the presence of polymorphisms in housekeeping genes, but not all organisms will have a sufficient amount of these to make such an assay meaningful, such as has been observed with *M. pneumoniae* (Degrange et al., 2009). In this case, other typing methods must be used.

MLVA is a typing scheme that is similar to MLST in some aspects but has a few distinct differences. This assay uses the presence of tandem repeats in the genome to type bacteria. As with MLST, a few gene targets are chosen based on their level of variation. The type for MLVA, however, is dependent on the number of tandem repeats in the given targets and not the exact sequence (Vergnaud and Pourcel, 2006). Since the sequences are not needed, this method is less expensive than MLST, as the number of repeats can be determined based on the size of the PCR products. MLVA still has its drawbacks, however, as the exact same typing scheme must be used to compare isolates as with MLST.

Emerging Tools for Identification and Characterization

High-resolution melting PCR

With the appropriate instruments and PCR reagents, additional information can be obtained in a high-resolution melting (HRM) analysis, an emerging diagnostic tool. Following a real-time PCR assay which incorporates an intercalating dye, a step can be added in which the DNA is slowly melted to determine the exact melting point of the strand. When the instrument reaches the T_M of the PCR product, the two strands of DNA will separate, releasing the dye. This temperature is constant for a given DNA sequence and is determined by its size and GC-content.

For HRM-PCR, instruments with improved temperature specificity and stability are needed along with specific fluorescent dyes without intercalation bias (Taylor et al., 2010). HRM is a very powerful tool that was originally used to distinguish between heterozygotes and homozygotes of a gene (Gundry et al., 2003). Now, the uses of HRM have expanded to include detection of single nucleotide polymorphisms, of DNA methylation, and antibiotic resistance markers as well as genotyping (Tamburro and Ripabelli, 2017). HRM has also been used for bacterial identification and differentiation (Winchell et al., 2010).

HRM has been used in this regard for *Mycoplasma* species as well. Two *Mycoplasma* species that affect poultry, *M. gallisepticum* and *M. synoviae*, have temperature sensitive vaccine strains, but often these are difficult to distinguish from pathogenic field strains. HRM-PCR has been used to rapidly distinguish between field strains and vaccine strains of these organisms, improving flock status determination (Ghorashi et al., 2013; Shahid et al., 2014). Differentiation between strains of the same *Mycoplasma* species has also been done, distinguishing temperature sensitive, non-temperature sensitive, and field strains of *M. gallisepticum* (Ghorashi et al., 2010). HRM has also been used to detect a mixed population of *M. gallisepticum*. A 2015 study showed that an HRM-PCR assay was able to detect two different strains of *M. gallisepticum* in the same sample at varying concentration ratios (Ghorashi et al., 2015). Lastly, HRM has been used as a tool for identification of unknown *Mycoplasma* species using a set of universal primers (Rebelo et al., 2011). This study was limited to isolates of ruminant, avian, and canine origin, but showed the broad application yet specific capabilities of HRM-PCR with *Mycoplasma* species.

MALDI-TOF Mass Spectrometry

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is a relatively recent technology that has become a widespread diagnostic tool.

MALDI was developed in the 1980s with the ability to ionize larger molecules like proteins and evolved into a tool used for bacterial identification (Singhal et al., 2015). The instruments used in this process contain a laser and a vertical vacuum tube with a detector at the top. Bacterial cultures or protein extracts are placed on a small metal plate and α-cyano-4-hydroxycinnamic acid (called matrix) is added. Once dry, the laser ionizes the proteins and they break away from the plate and rise through the vacuum to the detector. Based on the time the ionized proteins take to reach the top, spectral data are generated and these are compared to a library to identify the unknown organisms (Holland et al., 1996; Krishnamurthy and Ross, 1996). For each unknown isolate, a logarithmic score is generated based the amount of peaks that match and the intensity of those peaks (Bruker Daltonics, 2012). With a strong database, MALDI-TOF can be a valuable tool in bacterial identification. A 2010 reported the ability to distinguish 24 different species of *Staphylococcus* totaling over 1,000 isolates with 100% accuracy (Szabados et al., 2010). The initial cost of an instrument for MALDI-TOF mass spectrometry is high, but after this expenditure the cost per isolate can be as low as $0.43, making it a worthwhile investment for laboratories that can afford the instrument (Tran et al., 2015).

MALDI-TOF has been shown to be effective for determination of *Mycoplasma* species in addition to conventional bacteria. A significant respiratory pathogen of rats and mice, *Mycoplasma pulmonis*, was shown to be accurately identified using this system, even when compared to similar species (Goto et al., 2012). A 2013 study showed that MALDI-TOF was even able to correctly distinguish between two subtypes of *M. pneumoniae* for all 50 isolates tested (Pereyre et al., 2013). While more rapid tests not requiring a culture (such as PCR) may be preferred, in unique circumstances MALDI-TOF may still be a better option. For example, in 2014 MALDI-TOF was used on a clinical case to identify *Mycoplasma hominis,* a pathogen that

rarely causes brain abscesses. Ultimately, MALDI-TOF was able to quickly identify the pathogen and provide insight into the best treatment for the patient (Pailhories et al., 2014).

Conclusion

The Mollicutes are a group of bacteria that vary phenotypically and genetically from their more conventional prokaryotic cousins yet can still have significant impact in human medicine, veterinary medicine, and research. Many different species of *Mycoplasma* and *Ureaplasma* can cause disease in various animal hosts, and these diseases are harder to treat due to the structural nature of the Mollicutes. While many different identification methods exist, most require reagents specific to an individual species, making the diagnostic process much more expensive, time-consuming, and difficult. These and other methods require multiple passages of cultures to both isolate the organisms and then perform biochemical tests. Because of this, faster and cheaper tests are needed to improve the diagnostic process in order to quickly and inexpensively address mycoplasma issues in humans, animals, or cell culture.

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CHAPTER 3: COMPARISON OF MALDI-TOF MASS SPECTROMETRY AND HIGH RESOLUTION MELTING PCR FOR THE IDENTIFICATION OF *MYCOPLASMA BOVIS* ISOLATES

Abstract

Mycoplasma bovis is an important pathogen of cattle worldwide. Many different clinical manifestations of infection can occur, including respiratory disease, arthritis, and mastitis, causing heavy losses to the beef and dairy industries every year. Because *Mycoplasma* species are slow-growing and fastidious, traditional identification methods are not cost or time-effective, and improved methods are sought to improve the diagnostic process. High resolution melting polymerase chain reaction (HRM-PCR) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry are two relatively recent diagnostic tools that are rapid and inexpensive to perform, although equipment cost may be substantial. Eight field isolates and one reference strain of *M. bovis* were grown and used in both assays, followed by a comparison of the results. The HRM-PCR assay used universal *Mycoplasma* primers for the 16S-23S intergenic spacer region (IGSR). The resulting melt peak profiles of the field isolates were indistinguishable from the reference strain, indicating accurate identification. For MALDI-TOF mass spectrometry, each isolate was accurately identified on all replicates. Because both assays correctly identified all field isolates, our work shows that either assay could be used to identify an unknown *M. bovis* isolate. For future work, the MALDI-TOF library should be expanded to include spectra from more *Mycoplasma* species, and the HRM-PCR assay should be tested on other species of *Mycoplasma* to ensure that the melting profile of each species is sufficiently distinctive that they can be used for species differentiation.

Introduction

Mycoplasma bovis is an important pathogen to the beef and dairy industries, causing respiratory disease, arthritis, mastitis, and other infections as well (Maunsell et al., 2011). Few studies of its economic impact have been conducted, but it is estimated that in the United States up to $32 million per year is lost by the beef industry due to reduced weight gain, and up to $108 million per year is lost by the dairy industry because of mastitis (Maunsell et al., 2011). Identification of *M. bovis* and other *Mycoplasma* species in general can be an arduous task. While biochemical tests are the gold standard for conventional bacteria, these methods take weeks for the fastidious and slow-growing *Mycoplasmas.* In order to quickly diagnose cattle to prevent further spread of the infection, faster methods of identification are needed.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry is a tool that can be used to analyze the protein composition of bacteria (Holland et al., 1996; Krishnamurthy and Ross, 1996). In the last 15 years, this technology has been used to identify bacteria based on their protein profile created (Bernardo et al., 2002; Szabados et al., 2010). With regards to *Mycoplasma* species, MALDI-TOF has been used to identify clinically relevant human isolates (Pereyre et al., 2013; Pailhories et al., 2014) as well as isolates from a few animal species, including *M. bovis* (Goto et al., 2012; Pereyre et al., 2013).

Another diagnostic tool is high-resolution melting polymerase chain reaction (HRM-PCR). This technology can be used for a variety of purposes such as genotyping and strain differentiation of bacteria (Gundry et al., 2003; Winchell et al., 2010; Tamburro and Ripabelli, 2017). These applications have also been successfully implemented with viruses, fungi, and protists (Adaszek and Winiarczyk, 2010; Didehdar et al., 2016; Toubanaki and Karagouni, 2017). One benefit of HRM-PCR over traditional real-time PCR for pathogen identification is

that one set of primers can be used to distinguish between different species or different strains of the same species, increasing efficiency. Depending on the target for the assay, different species or strains will exhibit a unique melt profile. Much of the HRM-PCR work done with *Mycoplasma* species has been with poultry isolates to distinguish between vaccine strains and field strains of the same species (Ghorashi et al., 2010; Ghorashi et al., 2013; Shahid et al., 2014), though general strain differentiation work has been done as well (Jeffery et al., 2007; Ghorashi et al., 2015). Little HRM-PCR has been done with identification of *Mycoplasma* species, though the efficacy of such an assay using universal primers for canine, avian, and ruminant *Mycoplasmas* has been explored (Rebelo et al., 2011).

The aim of this study was to compare the accuracy of HRM-PCR and MALDI-TOF mass spectrometry in the identification of *M. bovis* isolates for diagnostic use. Nine isolates from a variety of sites on the animal and locations within the United States were used along with one reference strain, and the results of the two assays were compared.

Materials and Methods

Mycoplasma bovis strains and culture

Eight *Mycoplasma bovis* strains isolated at the Iowa State University Veterinary Diagnostic Laboratory and Jasper strain (ATCC 25025) in were used for this study (Table 3.1). These isolates were grown for 3-5 days in PPLO broth with horse serum (University of California, Davis, USA) and supplemented with 5 µg/mL cefoperazone inhibitor (Sigma-Aldritch, USA) for the HRM-PCR. They were then subcultured onto PPLO agar with horse serum (University of California, Davis, USA) and grown for 3-5 days for use in MALDI-TOF mass spectrometry. All isolates were grown at 37° C with 7.5% CO_2.

Table 3.1. *Mycoplasma bovis* isolates and their origin

Isolate	Animal Host	Site of Isolation	Year Isolated
B1	Cattle	Joint Fluid	2013
B2	Cattle	Ear Swab	2013
B3	Cattle	Lung	2014
B4	Cattle	Bulk Tank Milk	2014
B5	Cattle	Lung	2014
B6	Cattle	Joint Fluid	2014
B7	Cattle	Eye Swab	2015
B8	Bison	Lung	2016
B9 (Jasper)	Cattle	Milk	1966

DNA extraction and high-resolution melting PCR

Genomic DNA was extracted from 200 μL of culture by boiling for 10 minutes. The cells were then pelleted by centrifugation in a tabletop centrifuge at 15,871 g for 3 minutes. In order to mimic the high-throughput environment of a diagnostic laboratory, the amount of DNA in each extract was not standardized. Universal primers targeting the 16S-23S intergenic spacer region (IGSR) were used for this assay as previously described: F 5′-ACACCATGGGAGYTGGTAAT-3′ and R 5′-CTCCWTCGACTTYCAGACCCAAGGCAT-3′ (Rebelo et al., 2011). The Qiagen HRM PCR Master Mix (Qiagen, Netherlands) was used according to manufacturer's recommendations with 2.5 μL of DNA extract per well. Sterile water was used in place of the extract for the negative amplification control, and sterile media was extracted for the negative extraction control. The HRM-PCR was performed on a Qiagen RotorGene Q (Qiagen, Netherlands) using the cycling conditions previously described (Rebelo et al., 2011).

MALDI-TOF mass spectrometry

After growing for approximately 3-5 days, the *M. bovis* isolates were run in MALDI-TOF. For each strain, whole cells were spotted four times onto a single steel target and run

according to the manufacturer's recommendations (Bruker, Germany). The result is expressed as a logarithmic score ranging from 0 to 3 with a score of ≥2.0 considered acceptable for identification, and this score is based on the number of peaks that match along with their intensity (Bruker Daltonics, 2012).

16S-23S IGSR sequence analysis and statistics

Using the whole genome sequence available in GenBank under accession number CP002188, the sequences of the two 16S-23S intergenic spacer regions were analyzed. These 311 bp sequences were aligned via BLASTn to observe differences in the sequences. Dunnett's test for multiple comparisons was used to compare the melting temperatures of the field isolates to those of the reference strain for the HRM-PCR assay. MALDI-TOF mass spectrometry data was analyzed using a one-way ANOVA with Tukey's multiple comparison test.

Results

All of the nine *Mycoplasma bovis* isolates exhibited two melt peaks: the first at 76.25 ± 0.005° C and the second at approximately 80.94 ± 0.008° C. Additionally, the height of the second peak was one-third the height of the first peak (Figure 3.1). No significant difference between the melting temperatures was present when comparing the field strains to the reference strain for either the first peak or the second peak (Table 3.2). Every isolate was correctly identified as *Mycoplasma bovis* by the MALDI-TOF software, though the top scores ranged from 1.71 to 2.39. The Jasper reference strain had the highest score values, but it was only significantly higher than two of the eight field strains (Figure 3.2).

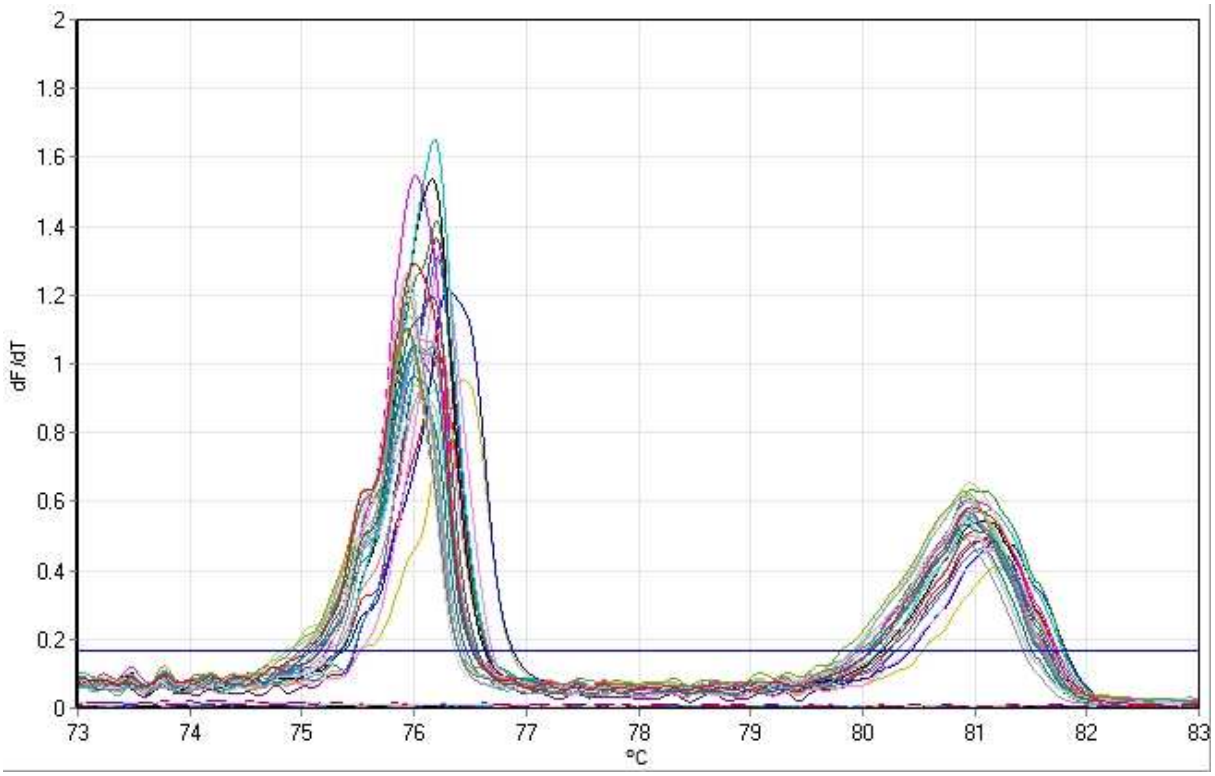

Figure 3.1. Identification of *Mycoplasma bovis* isolates by high-resolution melting PCR. HRM-PCR was done in triplicate for each isolate. The PCR threshold was determined automatically by the Qiagen Rotor-Gene Q Series Software.

Table 3.2. Mean melt peak temperatures* for *Mycoplasma bovis* isolates

Isolate	Mean Temperature (° C)	
	Peak 1	Peak 2
B1	76.255 ± 0.001	80.941 ± 0.001
B2	76.259 ± 0.005	80.950 ± 0.008
B3	76.257 ± 0.004	80.930 ± 0.005
B4	76.251 ± 0.003	80.940 ± 0
B5	76.256 ± 0.002	80.938 ± 0.002
B6	76.253 ± 0.002	80.945 ± 0.003
B7	76.251 ± 0.001	80.933 ± 0.001
B8	76.245 ± 0.001	80.937 ± 0.002
B9	76.249 ± 0.001	80.937 ± 0.001

* Data represents the mean of the three replicates ± SE.

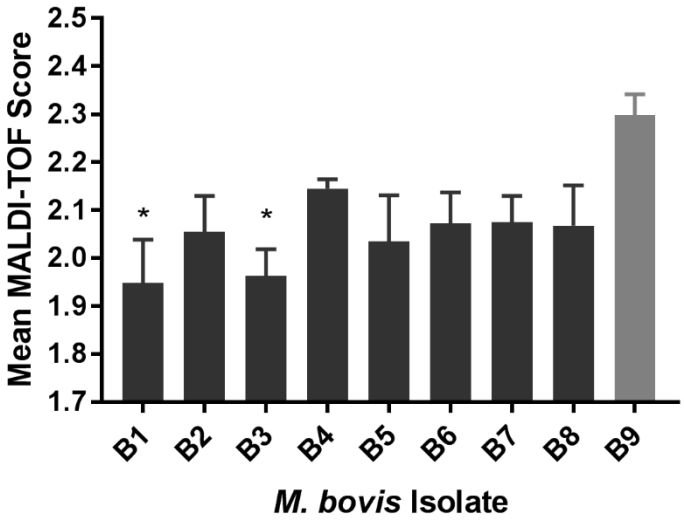

Figure 3.2. Mean MALDI-TOF score for each *Mycoplasma bovis* isolate. Dunnett's test was performed comparing the field isolates to B9 (grey), the reference strain. An asterisk indicates a p-value <0.05.

Discussion

In the HRM-PCR assay, two melting peaks were present for each *M. bovis* isolate, at temperatures consistent with previous findings for this assay (Rebelo et al., 2011). One possible explanation is that the *M. bovis* genome contains two copies of the rRNA genes, and if the sequences vary sufficiently, they could have resulted in different melting temperatures (T_M) (Wise et al., 2011). When comparing the DNA sequences of the two 16S-23S IGSR of the *M. bovis* PG45 genome, the sequences are almost identical, with only two deletions and one single nucleotide polymorphism in the second copy compared to the first copy. This would likely not be enough to change the T_M by almost five degrees, and even if it would, the brightness of the two peaks would be approximately equal as the two 16S-23S IGSR sequences are both 311 bp in length (Wise et al., 2011). An alternative explanation for the presence of two peaks is that the

sequences have two regions with significantly different GC content, resulting in a partial melt followed by a complete melt at a higher temperature. This second explanation seems to be more likely, as the second, higher T_M (80.94° C) of each isolate was less fluorescent, indicating that less dye was released and so less DNA remained to melt.

The MALDI-TOF scores for the reference strain of *M. bovis* tended to be higher than at least some of the field strains. This was expected, as spectra for that exact strain are present in the MALDI-TOF library used. While each organism was identified correctly by the MALDI-TOF software, the library used did not include a large number of *Mycoplasma* species. Spectra from only ten species other than *M. bovis* were present, and none of these were from closely related species such as *M. agalactiae*, *M. californicum*, or *M. fermentans* (Mattsson et al., 1994; Pettersson et al., 1996). Many of the scores were also between 1.7 and 2.0, indicating that the top scoring organism may not be acceptable for species-level identification, though other investigators have determined that scores above a 1.7 may be sufficient for certain *Mycoplasma* species including *M. bovis* (Pereyre et al., 2013). Without the addition of spectra of more closely related species, however, it is not possible to determine if the spectra for *M. bovis* in the present library are sufficient for consistent, accurate identification.

The two methods that were explored both resulted in accurate identification 100% of the time. The HRM-PCR assay found no difference between the melting peaks of the field isolates and the reference strain, and all replicates of all isolates in MALDI-TOF resulted in scores of 1.7 or greater. Both assays can be completed within just a few hours and, if using validated, universal primers for the HRM-PCR, both assays could be used for many different species, making them overall much less expensive than a separate assay for each individual organism. These tools are testing different aspects of the genetic code, however, and depending on the

circumstance, one test may be preferred. MALDI-TOF creates spectra from the proteins of the cultures while HRM-PCR generates a melt curve profile from the DNA. MALDI-TOF would find no difference between two isolates with varying silent mutations, as these do not affect the encoded amino acids, and for identification purposes, this would be preferred. As new, variant isolates of a species are discovered, additional spectra can be added to the database to improve its diagnostic capabilities. HRM-PCR relies on only one isolate per species as a control whereas MALDI-TOF needs spectra from multiple isolates of a species in order to get a well-rounded library of spectra of a given species. If the target sequence varies too much with a given species, however, HRM-PCR will not be able to identify that organism, and so the PCR targets must be extremely well-validated.

In the future, other organisms should be explored for the HRM-PCR assay to confirm that between *Mycoplasma* species the melt curve profile generated is unique, but within species it is the same. Additionally, the potential use of HRM-PCR directly of tissue samples should be investigated, as this would drastically decrease the time and cost required for identification. For MALDI-TOF, the library should be expanded to include spectra from many more *Mycoplasma* species, including those that are closely related and therefore might have similar protein profiles.

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CHAPTER 4: COMPARISON OF TWO HIGH-RESOLUTION MELTING PCR ASSAYS FOR THE IDENTIFICATION OF *MYCOPLASMA* SPECIES

Abstract

Mycoplasma and *Ureaplasma* species are significant veterinary pathogens, causing significant losses to producers of swine, cattle, and poultry every year. More rapid and inexpensive diagnostic tests are needed for these organisms in order to more quickly treat affected animals and prevent further losses. A high-resolution melting polymerase chain reaction (HRM-PCR) assay for the identification of unknown *Mycoplasma* species was developed and compared to a previously described assay. Nine different species were used in these assays: *Mycoplasma bovis, Mycoplasma canis, Mycoplasma hyorhinis, Mycoplasma hyosynoviae, Mycoplasma hyopneumoniae, Mycoplasma gallisepticum, Mycoplasma flocculare, Mycoplasma synoviae,* and *Ureaplasma diversum*, six of which had field strains in addition to the reference strains. The previously developed assay was able to accurately identify all isolates of *M. bovis* and *M. hyopneumoniae*, but only some isolates of the other organisms. It also did not amplify *M. gallisepticum* or *U. diversum*. The new assay identified all isolates of *M. bovis, M. hyopneumoniae,* and *M. canis* and most *M. hyorhinis*, but only some of the *M. hyosynoviae* and none of the *M. gallisepticum*. While HRM-PCR is a promising tool for identification of unknown isolates, exploration of many more species and alternative targets may be necessary for consistent, accurate results.

Introduction

Mycoplasma and *Ureaplasma* species, often simply Mycoplasmas, are significant veterinary pathogens, costing producers millions of dollars every year (Mohammed et al., 1987;

Maunsell et al., 2011; Holtkamp, 2014). These organisms are different from most bacteria in that they completely lack a cell wall. This defining feature also makes the resulting infections much more difficult to treat, as many common antibiotics target the cell wall of bacteria and so these will be ineffective against mycoplasmal infections (Razin, 1983). Because these bacteria are more difficult to treat, a fast diagnosis is key in order to provide the right treatments to infected animals. Mycoplasmas are slow-growing and fastidious organisms, however, some taking as long as 40 days to culture (Jensen et al., 1996). Traditional biochemical tests usually require two culturing steps, and so these take too long in a diagnostic setting (Goll, 1994). Additionally, while molecular assays such as polymerase chain reaction (PCR) are rapid, they are generally species-specific, and so multiple different tests are needed to identify unknown isolates, greatly increasing costs.

High-resolution melting PCR (HRM-PCR) is a recent diagnostic tool that functions like traditional real-time PCR, but has an extra melt step at the end. Additionally, less-biased fluorescent dyes are used in HRM-PCR in order to more accurately determine the melting point of the DNA amplicon (Taylor et al., 2010). After the assay is complete, a melt curve profile is generated for each PCR amplicon and serves as the defining feature of the assay. HRM-PCR has a very wide range of applications, from genotyping and single nucleotide polymorphism analysis to pathogen identification and characterization (Winchell et al., 2010; Tamburro and Ripabelli, 2017). For *Mycoplasma* species, HRM-PCR has been used for a few select purposes. Differentiating between vaccine strains and field strains has been explored primarily with poultry pathogens (Ghorashi et al., 2013; Shahid et al., 2014; Zhu et al., 2017). HRM-PCR has also been used as a tool for discrimination between different species of *Mycoplasma* and as a potential tool for identification (Rebelo et al., 2011; Al-Farha et al., 2018).

The aim of this study was to develop an HRM-PCR assay using universal primers for *Mycoplasma* and *Ureaplasma* species and compare this to a previously developed assay for the identification of unknown isolates. As each species would result in its own unique melt curve profile, an unknown isolate with the same as a control species would reveal its identity.

Materials and Methods

Strains

Eight different *Mycoplasma* and one *Ureaplasma* species were used in the HRM-PCR assays (Table 4.1). For each organism, one reference strain was used and for *Mycoplasma hyopneumoniae*, *Mycoplasma hyorhinis*, *Mycoplasma hyosynoviae*, *Mycoplasma bovis, Mycoplasma canis,* and *Mycoplasma gallisepticum*, field strains were also utilized, most originating from the Iowa State University Veterinary Diagnostic Laboratory (Table 4.2).

Media and Culture

Mycoplasma bovis and *M. canis* isolates were grown in PPLO broth with horse serum (University of California-Davis) for 3-5 days with 7.5% CO_2. The *M. gallisepticum* isolates were grown in Hayflicks broth (University of California-Davis) for 5-7 days with 7.5% CO_2. The *M. hyopneumoniae*, *M. hyorhinis*, and *Mycoplasma flocculare* isolates were grown in Friis broth with tight caps for 3-7 days. For the *M. hyosynoviae* isolates, PPLO broth with turkey serum and mucin (ThermoFisher Scientific, USA) was used, and these were grown with tight caps for 3-5 days. The *M. synoviae* strain was grown in PPLO broth with horse serum and NAD (ThermoFisher Scientific, USA) for 3-5 days with 7.5% CO_2. Lastly, the *Ureaplasma diversum* strain was grown in PPLO broth with horse serum and urea (ThermoFisher Scientific, USA) for 5-7 days. All isolates were cultured at 37° C.

Table 4.1. *Mycoplasma* and *Ureaplasma* species used and their associated diseases

Organism	Animal Hosts	Associated Infections and Conditions
Mycoplasma hyopneumoniae	Swine	Porcine enzootic pneumonia (Maes et al., 2018)
Mycoplasma hyorhinis	Swine	Arthritis, lameness, polyserositis (Wilberts et al., 2015)
Mycoplasma hyosynoviae	Swine	Arthritis, lameness (Wilberts et al., 2015)
Mycoplasma flocculare	Swine	None (Calcutt et al., 2015)
Mycoplasma bovis	Cattle	Respiratory disease, arthritis, mastitis, conjunctivitis, otitis media (Maunsell et al., 2011)
	Bison	Respiratory disease, lameness, mastitis (Janardhan et al., 2010)
Mycoplasma canis	Dogs	Urogenital disease, meningoencephalitis (L'Abee-Lund et al., 2003; Michaels et al., 2016)
	Cattle	Respiratory disease (Thomas et al., 2002)
Mycoplasma gallisepticum	Chickens	Chronic respiratory disease (Nascimento et al., 2005)
	Turkeys	Infectious sinusitis (Nascimento et al., 2005)
	Finches	Conjunctivitis (Luttrell et al., 1998)
Mycoplasma synoviae	Chickens	Infectious synovitis (Nascimento et al., 2005)
	Turkeys	Infectious synovitis (Nascimento et al., 2005)
Ureaplasma diversum	Cattle	Endometritis, infertility, abortion, weak calf syndrome (Mulira et al., 1992)

Table 4.2. Collected strains and their site of origin

Species	Organism ID	Animal Host	Specimen
Mycoplasma bovis	ATCC 25523	Cattle	Milk
M. bovis	B1	Cattle	Joint
M. bovis	B2	Cattle	Ear
M. bovis	B3	Cattle	Lung
M. bovis	B4	Cattle	Milk
M. bovis	B5	Cattle	Lung
M. bovis	B6	Cattle	Joint
M. bovis	B7	Cattle	Eye
M. bovis	B8	Bison	Lung
M. bovis	B9	Cattle	Milk
Mycoplasma canis	ATCC 19525	Dog	Throat
M. canis	C1	Dog	BAL
M. canis	C3	Dog	BAL
M. canis	C5	Dog	Unknown
Mycoplasma hyorhinis	ATCC 17981	Swine	Nose
M. hyorhinis	R1	Swine	Nose
M. hyorhinis	R2	Swine	Spleen
M. hyorhinis	R4	Swine	Lung
M. hyorhinis	R5	Swine	Lung
M. hyorhinis	R6	Swine	Lung
M. hyorhinis	R7	Swine	Eye
M. hyorhinis	R8	Swine	Fibrin
M. hyorhinis	R9	Swine	Fibrin
Mycoplasma hyosynoviae	ATCC 25591	Swine	Joint
M. hyosynoviae	S1	Swine	Joint
M. hyosynoviae	S2	Swine	Joint
M. hyosynoviae	S3	Swine	Joint
M. hyosynoviae	S4	Swine	Joint
M. hyosynoviae	S5	Swine	Joint
M. hyosynoviae	S6	Swine	Joint
M. hyosynoviae	S8	Swine	Eye
M. hyosynoviae	S9	Swine	Joint
Mycoplasma hyopneumoniae	ATCC 25934	Swine	Lung
M. hyopneumoniae	P1	Swine	Lung
M. hyopneumoniae	P2	Swine	Lung
M. hyopneumoniae	P3	Swine	BAL
M. hyopneumoniae	P4	Swine	Lung
Mycoplasma flocculare	ATCC 27399	Swine	Lung
Mycoplasma gallisepticum	ATCC 19610	Chicken	Trachea/Airsac
M. gallisepticum	G1	House Finch	Eye
M. gallisepticum	G2	House Finch	Eye
M. gallisepticum	G3	House Finch	Eye
Mycoplasma synoviae	ATCC 25204	Chicken	Joint
Ureaplasma diversum	ATCC 43321	Cattle	Lung

DNA Extraction and HRM-PCR-1

Magnetic bead DNA extraction was performed on a KingFisher Flex Purification System (ThermoFisher Scientific, USA) using 50 µL of each culture, with sterile media serving as a

negative extraction control. For the HRM-PCR assay previously developed (HRM-PCR-1), the two primers used targeted the 16S-23S intergenic spacer region (IGSR) and were: F 5′-ACACCATGGGAGYTGGTAAT-3′ and R 5′-CTCCWTCGACTTYCAGACCCAAGGCAT-3′. The Qiagen HRM PCR Master Mix (Qiagen, Netherlands) was used with 0.5 μM of the forward and reverse primers. To each well, 22.5 μL of the complete master mix and 2.5 μL of DNA extract was added. Sterile water was used in place of the extract for the negative amplification control. The HRM-PCR-1 assay was performed on a Qiagen RotorGene Q (Qiagen, Netherlands) in duplicate using the cycling conditions previously described (Rebelo et al., 2011).

HRM-PCR-2

For the newly developed HRM-PCR assay (HRM-PCR-2), DNA was extracted as above, and the PCR master mix was prepared as HRM-PCR-1 but with different primers. The new primers were: F 5'-CCTACGAGAACGTCGGGRT-3' and R 5'-AGACCCAAGGCATCCACYA-3'. In development of HRM-PCR-2, the reference strains were run in triplicate on the same instrument as HRM-PCR-1 with the following cycling conditions: 95° C for 5 min, followed by 40 cycles of 94° C for 30 sec, 53° C for 30 sec, and 72° C for 60 sec, then 72° C for 10 min, 95° C for 60 sec, and 40° C for 60 sec. The HRM analysis immediately followed the PCR and consisted of a melt from 60° C to 95° C rising 0.05° C/sec. To test the field strains, all cultures, including the reference strains, were run in duplicate following the same conditions as above.

Analysis and Statistics

Following PCR, the products for all *M. hyosynoviae* and *M. gallisepticum* strains were sequenced by Sanger sequencing. The sequences were analyzed using DNASTAR Lasergene 13

and MEGA X. HRM-PCR-2 data was analyzed using the Qiagen Rotor-Gene Q Series Software (Qiagen, Netherlands). For the HRM analysis, *M. bovis* was chosen as the normalization organism, comparing all other melt curve profiles to the *M. bovis* profile. The HRM-PCR-2 melt peaks of the reference strains were first compared to each other using a one-way ANOVA with Tukey's multiple comparison test. Then for both HRM-PCR-1 and HRM-PCR-2, melt peaks from each field isolate were compared to those of each control also using a one-way ANOVA with Tukey's multiple comparison test.

Results

In HRM-PCR-1, each isolate gave two or three melt peaks ranging from about 72.6° C to 82.5° C (Table 4.3). Two species, *M. gallisepticum* and *U. diversum*, did not amplify with this assay at all. The melt peak temperatures of each respective peak (first, second, third) were compared between the reference strains and field strains. Of the field isolates tested, all *M. bovis* and *M. hyopneumoniae* strains were correctly identified, and 2/3 *M. canis*, 2/8 *M. hyorhinis*, and 3/8 *M. hyosynoviae* strains were correctly identified. The HRM analysis showed distinct differences between standards but was not able to further identify field isolates (Figure 1A).

For the HRM-PCR-2 assay with the reference strains, each species gave one to three peaks with melt temperatures ranging from approximately 72.3° C to 76.4° C (Table 4.3). For the statistical analysis, data from each species' first peak was compared followed by a comparison of data from the second peak. No analysis was done on third peaks, as only *M. hyopneumoniae* had a third peak. All of the first peaks were significantly different from each other ($p < 0.05$) except for *M. hyorhinis* and *U. diversum*. For the second peaks, only *M. hyorhinis* and *M. gallisepticum* were not significantly different. The difference curve of the HRM analysis showed distinct differences between each of the species tested (Figure 4.1B). When comparing just the melting

temperatures of the field strains, many of the isolates were not correctly identified by one peak alone. When taking into account all peaks and the melting temperatures of the peaks, however, all *M. bovis* and *M. hyopneumoniae* isolates were correctly identified. With the addition of the HRM analysis, 7/8 *M. hyorhinis* isolates, all *M. canis* isolates, and 2/8 *M. hyosynoviae* isolates were identified correctly. None of the *M. gallisepticum* field isolates were correctly identified with this HRM-PCR assay (Table 4.4).

Table 4.3. Melt peak temperatures* of the reference strains

Reference Species	**HRM-PCR-1 T_M**			**HRM-PCR-2 T_M**		
	Peak 1	**Peak 2**	**Peak 3**	**Peak 1**	**Peak 2**	**Peak 3**
M. bovis	75.73 ± 0.010° C	80.92 ± 0.025° C	–	75.58 ± 0.020° C	–	–
M. canis	75.80 ± 0.013° C	82.51 ± 0.005° C	–	75.05 ± 0° C	75.41 ± 0.003° C	–
M. hyorhinis	74.24 ± 0° C	81.95 ± 0.005° C	–	73.94 ± 0.008° C	76.21 ± 0.005° C	–
M. hyosynoviae	76.01 ± 0.028° C	82.22 ± 0.025° C	–	75.76 ± 0.028° C	–	–
M. hyopneumoniae	72.68 ± 0° C	74.66 ± 0.008° C	82.24 ± 0.003° C	72.8 ± 0.010° C	74.22 ± 0.030° C	75.70 ± 0.025° C
M. flocculare	74.22 ± 0.020° C	82.51 ± 0.013° C	–	73.33 ± 0.005° C	–	–
M. gallisepticum	–	–	–	74.24 ± 0° C	76.21 ± 0° C	–
M. synoviae	74.60 ± 0.010° C	77.96 ± 0.003° C	82.56 ± 0.008° C	74.53 ± 0.008° C	76.83 ± 0.043° C	–
U. diversum	–	–	–	73.99 ± 0.013° C	75.02 ± 0° C	–

* Melt peak temperatures were done in duplicate. Data represents mean temperature ± SE. A dash indicates that no peak was present.

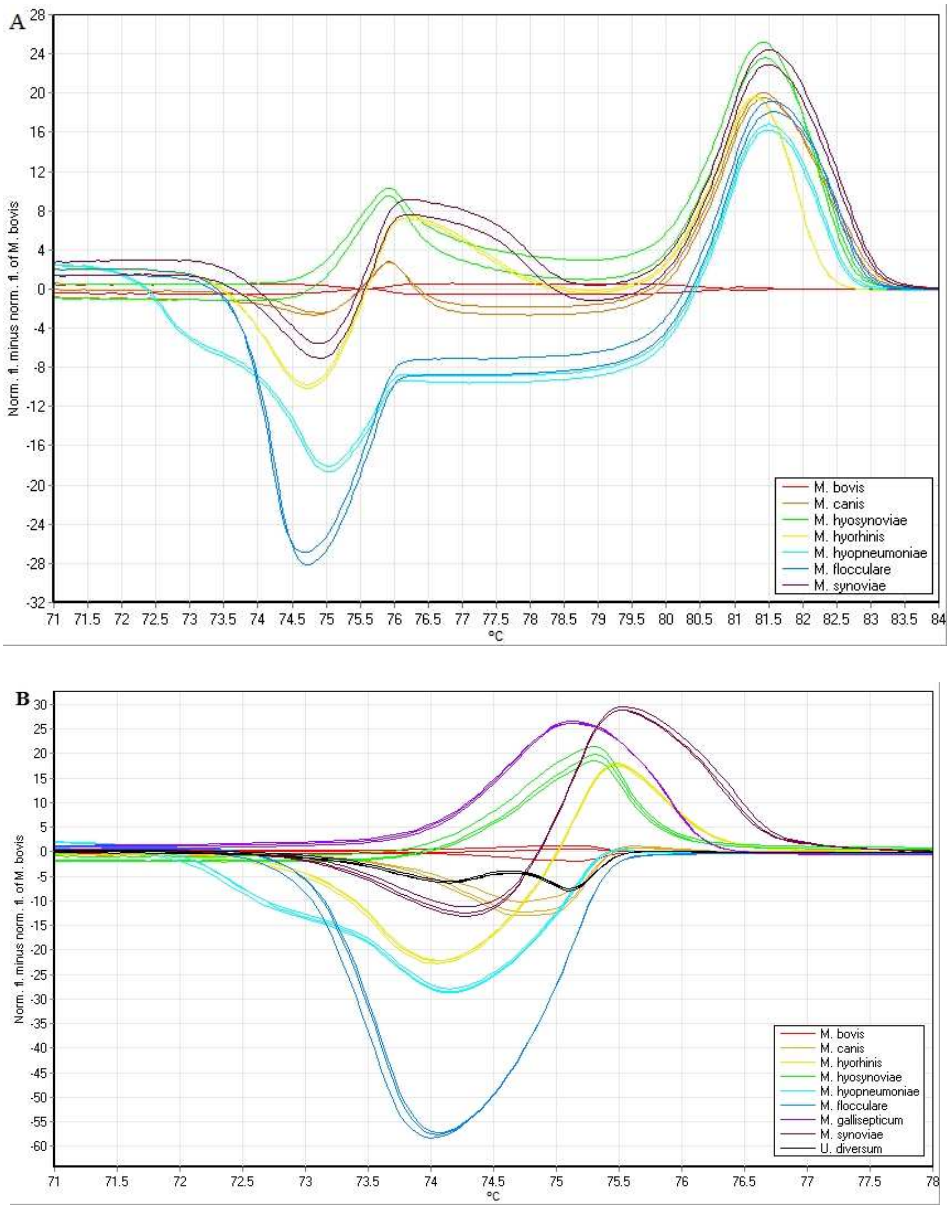

Figure 4.1. Difference curve for the high-resolution melt analyses of *Mycoplasma* and *Ureaplasma* reference strains. The difference curves for both HRM-PCR-1 (A) and HRM-PCR-2 (B) were generated using *M. bovis* as the fluorescence baseline.

The sequence analysis of the *M. gallisepticum* isolates showed that while the three field isolates were nearly identical, the reference strain had 12 single nucleotide polymorphisms (SNPs) and a 4 bp insertion throughout the 705 bp region, compared to the field isolates. The

sequence analysis of the *M. hyosynoviae* isolates showed only 7 SNPs throughout the 270 bp region, though these did not appear to be restricted to the reference strain. Generation of a maximum-likelihood phylogenetic tree showed that there were four distinct sequence groups for the field isolates with the reference strain in a fifth group (Figure 4.2).

Discussion

Of the five species of field isolates that amplified in the HRM-PCR-1 assay, only two were identified with 100% accuracy. Only seven of the nine species tested were amplified in this assay, though one of those that did not amplify was a *Ureaplasma* species and the primers were not designed with those in mind. The assay was originally developed for use with *Mycoplasma* species of ruminant, canine, and avian hosts, but the PCR did not work with *M. gallisepticum*, an important avian pathogen (Rebelo et al., 2011).

As expected, the reference strains of all nine species each gave a distinct melt curve profile in the HRM-PCR-2 assay. Additionally, this assay was able to correctly identify all of the *M. bovis* and *M. hyopneumoniae* isolates and most of the *M. hyorhinis* and *M. canis* isolates. Only 25% of the *M. hyosynoviae* isolates were correctly identified, and none of the *M. gallisepticum* isolates were correctly identified. The one *M. hyorhinis* strain that did not correctly identify was isolated from the eye of a pig. It has been shown that some *M. hyorhinis* strains that are associated with conjunctivitis vary serologically compared to other strains of *M. hyorhinis*, and so it is possible that the unidentifiable isolate lies within this subgroup (Friis, 1976). Alternatively, one strain of *M. bovis* was isolated from a bison, and it has been shown that isolates from bison and cattle are also genetically distinct (Register et al., 2015).

Table 4.4. High-resolution melt analysis identification result

Organism ID	Isolate	HRM-PCR-1 ID	HRM-PCR-2 ID
B1	*M. bovis*	*M. bovis*	*M. bovis*
B2	*M. bovis*	*M. bovis*	*M. bovis*
B3	*M. bovis*	*M. bovis*	*M. bovis*
B4	*M. bovis*	*M. bovis*	*M. bovis*
B5	*M. bovis*	*M. bovis*	*M. bovis*
B6	*M. bovis*	*M. bovis*	*M. bovis*
B7	*M. bovis*	*M. bovis*	*M. bovis*
B8	*M. bovis*	*M. bovis*	*M. bovis*
B9	*M. bovis*	*M. bovis*	*M. bovis*
C1	*M. canis*	*M. canis*	*M. canis*
C3	*M. canis*	None*	*M. canis*
C5	*M. canis*	*M. canis*	*M. canis*
R1	*M. hyorhinis*	None	*M. hyorhinis*
R2	*M. hyorhinis*	*M. hyorhinis*	*M. hyorhinis*
R4	*M. hyorhinis*	None	*M. hyorhinis*
R5	*M. hyorhinis*	None	*M. hyorhinis*
R6	*M. hyorhinis*	None	*M. hyorhinis*
R7	*M. hyorhinis*	*M. hyorhinis*	None
R8	*M. hyorhinis*	None	*M. hyorhinis*
R9	*M. hyorhinis*	None	*M. hyorhinis*
S1	*M. hyosynoviae*	*M. hyosynoviae*	*M. hyosynoviae*
S2	*M. hyosynoviae*	None	None
S3	*M. hyosynoviae*	*M. hyosynoviae*	*M. hyosynoviae*
S4	*M. hyosynoviae*	None	None
S5	*M. hyosynoviae*	None	None
S6	*M. hyosynoviae*	None	None
S8	*M. hyosynoviae*	*M. hyosynoviae*	None
S9	*M. hyosynoviae*	None	None
P1	*M. hyopneumoniae*	*M. hyopneumoniae*	*M. hyopneumoniae*
P2	*M. hyopneumoniae*	*M. hyopneumoniae*	*M. hyopneumoniae*
P3	*M. hyopneumoniae*	*M. hyopneumoniae*	*M. hyopneumoniae*
P4	*M. hyopneumoniae*	*M. hyopneumoniae*	*M. hyopneumoniae*
G1	*M. gallisepticum*	N/A	None
G2	*M. gallisepticum*	N/A	None
G3	*M. gallisepticum*	N/A	None

* Isolates without an ID did not have melt peaks or a melt curve profile that matched a reference strain.

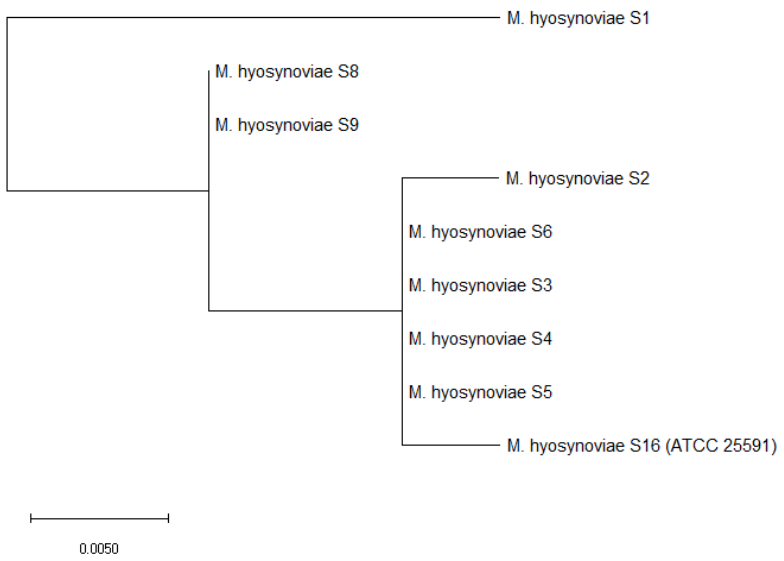

Figure 4.2. Maximum-likelihood phylogenetic tree of *M. hyosynoviae* strains based on 16S-23S rRNA intergenic spacer region. Phylogenetic tree generated in MEGA X software.

None of the three *M. gallisepticum* isolates were correctly identified using this method, however all three isolates originated from house finches while the reference strain originated from a chicken. The melt curve profiles of these three house finch isolates were indistinguishable from each other. This indicates that house finch isolates may be members of a separate group of *M. gallisepticum,* as was seen in the sequencing of the PCR products. This reasoning is supported by previous phylogenetic studies that have shown that *M. gallisepticum* isolates from house finches are distinct from isolates from poultry (Delaney et al., 2012). The *M. hyosynoviae* isolates also identified poorly. Sequencing results indicated that there were SNPs throughout the sequences, accounting for 2.6% of differences compared to 1.7% with *M. gallisepticum*. The phylogenetic tree separated the *M. hyosynoviae* isolates into five groups. Interestingly, when comparing the HRM data of the field strains to each other as opposed to the reference strain, isolate S1 was significantly different from S2 and S9 with no other significant differences,

differing from the groupings of the sequence data. Most likely this is due to differences in calculations, as the HRM identification has a range of acceptable statistical difference whereas the phylogenetic tree considers every individual nucleotide difference. Some subgroups within a species may only require a single control, while other subgroups within a species may not identify correctly using this method without a separate reference strain for that subgroup.

The two HRM-PCR assays were both able to correctly identify the *M. bovis* and *M. hyopneumoniae* isolates. Those two species have the greatest clinical disease implications of *Mycoplasma* species of their respective hosts, however other significant pathogens were not properly identified. The newly developed assay was able to identify most *M. hyorhinis* isolates, however neither assay was able to identify the *M. hyosynoviae* isolates. This suggests that the 16S-23S IGSR of *M. hyosynoviae* is not well conserved between species. It is worth noting, however, that while these isolates could not be confirmed as *M. hyosynoviae*, they were not misidentified as another species. The second assay also gave different melt curve profiles for each species. All of the isolates in HRM-PCR-1 had at least two melt peaks, while one-third of the species had only one peak in HRM-PCR-2. While the two assays produced amplicons of similar size, the slight differences in sequence were enough to cause drastic changes in the melt curve profiles of the organisms.

The HRM-PCR-2 assay worked on all nine species tested, while the HRM-PCR-1 assay did not, indicating that the former can be used on a wider array of species. While neither assay was able to accurately identify all isolates, the HRM-PCR-2 assay worked correctly for most and so shows the most promise as a diagnostic tool for identification of unknown isolates. Future work should look at a much wider variety of species and include field isolates for each of those

to ensure the homogeneity of melt curves within species. Additionally targets other than the 16S-23S IGSR should be explored for use in an HRM-PCR assay.

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CHAPTER 5: GENERAL CONCLUSIONS

MALDI-TOF mass spectrometry and HRM-PCR are two tools that have been shown to provide rapid, inexpensive, and accurate diagnosis for various pathogens. For *Mycoplasma* infections, this is key in order to apply the proper treatment regimen. The purpose of these studies was to determine which assay, if any, was superior, and then further explore the use of HRM-PCR as a tool for identification of *Mycoplasma* and *Ureaplasma* species.

When comparing MALDI-TOF with HRM-PCR, both assays were able to accurately and consistently identify *Mycoplasma bovis*. As neither assay was superior, it was determined that either could be used for that single species. The two HRM-PCR assays were both able to identify some species, however the new assay appeared to be superior. Neither assay was able to accurately identify all species tested, however. HRM-PCR-2 was unable to identify isolates that may be members of subgroups within their species. Only a few isolates of *Mycoplasma hyosynoviae* were properly identified, and these isolates were not known to be members of any subgroup, suggesting that perhaps alternative targets might prove superior to the 16S-23S IGSR for an HRM-PCR assay for swine specimens. As three of the species tested had only one isolate (the reference strain), the effectiveness of HRM-PCR on these species cannot be determined, only that other species are not misidentified as them. Additionally, more field isolates of the various species need to be included to fully evaluate the effectiveness of the HRM-PCR.

Because both types of identification assays tested require libraries of known positives for comparison, additional species are needed to ensure that false identification does not occur. Future work should focus on expanding the library of spectra for MALDI-TOF to include a broader range of *Mycoplasma* species. Work with HRM-PCR should explore other conserved regions amongst *Mycoplasma* species in order to find better targets, and more isolates of more

species should be tested with the new 16S-23S IGSR primers to confirm the specificity of the melt curve profiles. In addition, the use of HRM-PCR directly on tissue samples should be explored in order to reduce time and costs.